Mycopath (2014) 12(2): 77-82 **Research Article**

Isolation of a putative invertase gene from the xerophilic *Aspergillus niger* GH1 strain

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Abstract

Invertase catalyzes the hydrolysis of sucrose into glucose and fructose and represents a biocatalyst of great importance in food industry. Some xerophilic fungal strains have a great potential to increase enzymatic production. In the present study, five xerophilic fungal strains (*Penicillium pinophilum* EH2, *P. purpurogenum* GH2, *P. citrinum* ESS, *Aspergillus niger* GH1 and *A. fumigatus* GS) previously reported as invertase producers were used for isolation and cloning of an invertase gene. A fragment of 1770 bp was isolated and cloned, which corresponding to invertase gene from *A. niger* GH1. On the other hand, multi-banding was observed from invertase gene from other strains and invertase gene could not be isolated. Results suggested that invertase gene and amino acid sequence exhibited 97% and 99% similarity to invertase from *A. niger* B60 and *A. kawachii* IFO 4308, respectively. Therefore, *A. niger* GH1 could be a good alternative for invertase production.

Key words: β-D-fructofuranosidase, cloning, fructooligosacharides, *Aspergillus niger, Penicillium.*

Introduction

Invertases or β-fructofuranosidases (Ffase) (EC 3.2.1.26) catalyze the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides, including hydrolysis of sucrose into glucose and fructose. These enzymes are used in beverage and confectionary industries (Kumar *et al*., 2001). In addition, invertase also catalyze synthesis of fructo-oligosaccharides (FOS), which are promising ingredients for functional foods since they act as "prebiotics", and exert a beneficial effect on human health, participating in the prevention of cardiovascular diseases, colon cancer, and osteoporosis (Linde *et al*., 2009; Kurakake *et al*., 2010). Industrially, invertases have been produced in submerged culture (SmC) using *Saccharomyces cerevisiae*. However, filamentous fungi, particularly *Aspergillus niger* strains secrete large amounts and varieties of enzymes, including invertase. In recent years, different xerophilic fungal strains were isolated from Mexican semi-desert and characterized as invertase producers (Veana *et al*., 2011; Flores-Gallegos *et al*., 2012). However, their nucleotide sequences have not been determined. Since, Boddy *et al*. (1993) described the sequence of invertase gene from *A. niger* B60, few studies have been reported about the use of heterologous expression system for increase the productivity of this enzyme. Present study was designed to analyze and describe the invertase nucleotide and amino acid sequence isolated from *A. niger* GH1. In addition, genetic relationship with other sequences of invertase was established.

Materials and Methods

Xerophilic fungal strains, host and vectors

Penicillium purpurogenum GH2, *P. pinophilum* EH2, *P. citrinum* ESS, *A. niger* GH1 and *A. fumigatus* GS from the fungal collection of the Food Research Department-School of Chemistry-UAdeC (Cruz-Hernández *et al*., 2005) were used in this study. The pTOPO vector (10 ng μL^{-1}) of TOPO TA cloning kit with pCR 2.1-TOPO, the pUC19 vector (10 pg μL^{-1}) and electrocompetent *Escherichia coli* TOP10 cells were purchased from Invitrogen (San Diego, CA). PDA and LB agar Miller media were acquired from DIXON and US Biological, México. Tween 80 (Amresco, Ohio) and Wizard® SV Gel and PCR Clean-Up System from Promega (Madison, WI) were used. All chemicals were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO) or from Productos Químicos Monterrey (Monterrey, Nuevo León, México).

Media and culture conditions

For each fungus, 30 mL of Potato-Dextrose-Agar (PDA) were placed in 250 mL flasks and inoculated with 100 µL of spores. Flasks were incubated at 30 °C for 4 days. The spores were harvested with sterile 0.1% Tween 80 (v/v) modified Czapek-Dox medium with the following composition (g L^{-1}): $NaNO_3$ (7.65); KH_2PO4 (3.04); $MgSO_4.7H_2O$ (1.52); KCl (1.52) and glucose to produce mycelium. Erlenmeyer flasks (with 100 mL of modified Czapek-Dox medium) were inoculated with 200 µL of harvested spores and incubated at 30 °C under constant agitation (150 rpm) for 4 days to obtain the mycelium. Subsequently, the biomass was washed three times with sterilized distilled water and frozen at -70°C in 50 mL Falcon tubes until DNA extraction.

PCR of invertase gene

DNA extraction was performed using TES extraction buffer (50 mM pH 7.5 Tris-HCl, 20 mM EDTA and 1% SDS) as reported by Barth and Gallardin (1996). Some modifications were realized; the addition of PEG was eliminated from the technique. On the other hand, washes with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) were performed and, addition of 7.5 M ammonium acetate and cold 100% ethanol (v/v) was included.

PCR reaction was as follow: 17.5 µL of sterile deionized distilled water, 2.5 µL 10X buffer, 1.5µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of 200 mM of each oligonucleotide, 0.5 µL of Taq polymerase (Invitrogen, San Diego, CA) and 1.5 µL of template DNA (200 ng). PCR amplification of the invertase gene was realized in an Axigen Maxygene thermocycler (Union City, CA USA) with the next conditions: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C (Inv oligonucleotides) and 52 °C [Inv2, Inv3 and Inv4 oligonucleotides] for 1 min and extension at 72 °C for 1.5 min. Then, a final elongation at 72 °C for 5 minutes was done. Primers were designed using invertase sequences from *A. niger* deposited in GenBank of NCBI. Primer-blast of NCBI and Oligoanalyzer 3.1 tools were used for this purpose. The oligonucleotides designed in the present study were:
Inv1-F [5'-atgaagcttcaaacggcttccgta-3'],
Inv1-R [5'-tcaccgaacccaagtactcaacg-3'],
Inv2-F [5'-caatttccccgaggatacgg-3'],
Inv2-R [5'-tactacttaggcatcacctcaagac-3'],
Inv3-F [5'-atggagcggcaaactagc-3'],
Inv3-R [5'-catgactctctgattctagc-3'],
Inv4-F [atgaagctcaccactaccac-3'],
Inv4-R [5'-cctgcccgccccgttgacca-3'].

PCR products were purified using Wizard® SV Gel and PCR Clean-Up System.

Cloning of invertase gene and transformation in *E. coli* TOP10

The invertase gene was cloned in pTOPO according to manufacturer's instructions (Invitrogen, San Diego, CA). The expression plasmid constructs were named pTOPO-InvGH1. Then, 2 µL (125 ng) of constructed plasmid were mixed with electrocompetent *E. coli* TOP10 cells, which were electroporated in 2 mm cuvette in ECM 399 electroporation system (Holliston MA) at 2450 V at 6 ms. Transformed cells (50 µL) were placed on Petri dish with LB agar added with ultrapure X-Gal (40 mg mL^{-1}) and ampicillin (50 µg mL^{-1}). Plates were incubated at 37 °C for 16 hours. The positive (10 pg μL^{-1} pUC19 DNA) and negative (distilled desionized sterile water) controls were used.

Then, positive clones (white colonies) were randomly selected for plasmid DNA isolation. Plasmids were grown in 2 mL of broth LB added with ampicillin (50 µg μL^{-1}) and were incubated at 37 °C, and stirred to 150 rpm for 16 hours. Cells were centrifuged at 21900 g for 1 min a 4 °C. After that, plasmid DNA was obtained by alkaline lysis with SDS Mini preps, as reported by Sambrook and Russel (2001) with some modifications. Plasmid recovery was performed with 7.5 M ammonium acetate and cold 100% (v/v) ethanol addition. Immediately, the plasmid DNA was amplified by PCR using the Inv1-F and Inv1-R oligonucleotides.

Invertase gene sequence analysis

Fragments from pTOPO-InvGH1 were sequenced at LANGEBIO (National Laboratory of Genomics for Biodiversity) of CINVESTAV-Irapuato (Mexico) by Sanger Method. The nucleotide sequences were analyzed and an *in silico* translation was performed with Bioedit 7.0 program. After that, alignments were realized using blastn and blastp of NCBI, the sequences with high identity (94-99%) were selected and a Neddleman-Wunsch global sequence alignment was realized.

A phylogenetic analysis was conducted in MEGA6; the evolutionary history was inferred using the Maximum Parsimony method. The conserved domains were obtained using Conserved Domain Database of NCBI and Pfam.

Molecular model from invertase from *A. niger* GH1 was constructed in PDB-sum. After that, signal peptide was predicted using signal 4.0 and molecular weight, pI, instability index were obtained with protparam tool of Expasy using the mature protein. Finally, the invertase gene Ifv was deposited in NCBI database under the accession number HQ450381 and protein sequence as ADR80690.1.

Results and Discussion

Recently, a xerophilic *A. niger* GH1 strain was described as an invertase producer under submerged (Veana *et al*., 2011) and solid-state cultures (Flores-Gallegos *et al*., 2012). In this study, amino acid sequence for the *A. niger* GH1 invertase was predicted. A single fragment of 1770 bp was obtained after PCR using genomic DNA from *A. niger* GH1. This fragment corresponded to an invertase gene reported previously (Boddy *et al*., 1993). On the other hand, multi-banding was observed using DNA from the others strains, probably because oligonucleotides were designed using invertase sequence from *A. niger*. For this reason, invertase gene fragments from *Penicillum* species could not be isolated and cloned in *E. coli*.

The invertase gene from *A. niger* GH1 (Fig. 1a) was cloned and its identification was corroborated by PCR (Fig. 1b) and the gene was named *Ifv*. According to nucleotide sequences analysis, 94-95% similarity to precursor Suc1 from *A. niger* CBS 513.88 was detected (Pel *et al*., 2007; GenBank accession no. XM_001393172.1) and beta-D-fructofuranosidase gene from *A. niger* IBT10sb (Somiari *et al*., 1997; GenBank accession no. AF029359.1, respectively). Boddy *et al*. (1993) reported studies of invertase gene from *A. niger* B60. The invertase gene (*Suc1*) was obtained from this strain, with a length of 1767 bp. In other studies, invertase gene (*Suc1*) was isolated from *A. niger* B60 and expressed in *Trichoderma reseei (*Bergès *et al*., 1993). However, the efficiency transformation was very low. In addition, the cellular multiplication time was larger than yeast and bacteria. On the other hand, β-fructofuranosidase gene (1.7 kb) from *A. niger* IBT10sb has been obtained and expressed in *E. coli*, which is similar to *Suc1* (Somiari *et al.*, 1997). However, the use of this system is very inefficient for protein secretion into the culture medium and toxicity is presented (Broadway, 2012).

According to maximum parsimony analysis of taxa, the consistency index was 0.92 (0.83), which measures the amount of homoplasy. This parameter must be at 1.0, indicating the homoplasy absence. The retention index was 0.78 (0.78) and measures the amount of synapomorphies (shared homologies between two or more taxa), with a range from 0 (no fit) to 1 (perfect fit). Composite index is 0.71 (0.65). These values are for all sites and parsimony-informative sites (in parentheses). The degree of relatedness between the genes coding for invertase was observed (Fig. 2) and divergence among invertase sequences may be due to alternative splicing and/or insertions through the time.

The amino acid sequence from *A. niger* GH1 presented high identity with invertases from *A. kawachii* IFO 4308 (Futagami *et al*., 2011; GenBank accession no. GAA88101.1), *A. niger* IBT10sb (Somiari *et al*., 1997; GenBank accession no. AAC08047.1), *A. niger* CBS 513.88 (Pel *et al*., 2007; GenBank accession no CAK45778.1), *A. niger* B60 (Boddy *et al*., 1993; GenBank accession no. S33920) with values of 93, 95, and 97%, respectively. In addition, the maximum identity of 100% was demonstrated that was compared the amino acids 373-557 region of *A. niger* GH1 reported previously by Flores-Gallegos *et al*. (2012) (GenBank accession no. AFU93429.1) and the sequence obtained in the present study.

In the global alignment, invertase from *A. niger* GH1 showed 9 conservative differences (27Ile→Val, 73Ala→Ser, 100Gln→Lys, 104Arg→Gln, 128Asn→Ser, 143Phe→Tyr, 244Tyr→Phe, 291Thr→Ser, 560Val→Ile) and 7 non-conservative (224Glu→Gly, 237Ser→Pro, 300Leu→Gln, 318Thr→Asn, 459Gly→Asp, 507His→Leu, 559Glu→Ala) compared with *A. niger* B60 invertase (Boddy *et al*., 1993). However, sequence of *A. kawachii* IFO 4308 (Futagami *et al*., 2011) has 39 extra residues at the C-terminus. If these region is removed, the highest identity (99%) is observed with invertase sequence from *A. niger* GH1. In the global alignment with *A. kawachii* IFO 4308, 8 differences were observed, 5 are conservative (27Ile→Val, 104Arg→Gln, 143Phe→Tyr, 560Val→Ile and 576Ile→Val) and 3 are non-conservative (328Thr→Asn, 427Ser→Gly, 559Glu→Ala). These positions not are associated to substrate-binding and catalytic triad amino acid sequences.
According to Pfam collection of protein families, a fragment of a glycosyl hydrolase family 32. Invertases, inulinases, levanases and other hydrolyzing-fructose enzymes have been included in this family, along with enzymes displaying transglycosylating activities.

GH32 N-terminal and GH32 C-terminal domains were identified in invertase sequence from *A. niger* GH1 (Residues. 91 to 401 and 485 to 581, respectively). In addition, 3 of 3 residues were detected that composed active site: Asp (D64), Asp (D194) and Glu (E 271), and 8 of 8 residues corresponding to substrate binding: Gly (G63), Asp (D64), Leu(L82) Phe (F121), Asp (D122), Arg (R193), Asp (D194) and Glu (E271). Residues Asp (D64), Asp (D194) and Glu (E271) have been described as the putative catalytic nucleophile, transition-state stabilizer, and general acid/base catalyst, respectively (Alberto *et al*., 2004). The molecular model from *A. niger* GH1 invertase was constructed using as template a fructosyl transferase from *A. japonicus (*Chuankhayan *et al*., 2010; PDB code 3LDK) with 64.3% of identity, including residues 15 to 574 (Fig. 3). The characteristic tridimensional structure was observed; two distinct domains forming a five-bladed β-propeller fold and a β sandwich structure.

Later, a signal peptide of 24 amino acids (MKLQTASVLLGSAAAASPSMQTRA) and a mature protein of 565 amino acids having a molecular weight of 61.39 kDa and pI of 4.81 was identified as well. Molecular weight and pI values, ranges from 75-340 kDa and 4.4-5.4, from *A. niger* have been reported previously (**S**chomburg *et al*., 2002)**.** Differences with the calculated molecular weight can be attributed to enzyme interactions with others molecules (carbohydrates, mainly) during its production. The enzyme is classified as stable according to instability index (25.81).

Conclusions

The nucleotide and amino acid sequences of an invertase from the xerophilic *A. niger* GH1 strain were described. The strain is well adapted to the Mexican Semi-Desert extreme conditions. The relatedness among the genes coding for invertase were determined. The nucleotide (97%) and amino acid sequences (99%) are more similar to invertase from *A. niger* B60 and *A. kawachii* IFO 4308.

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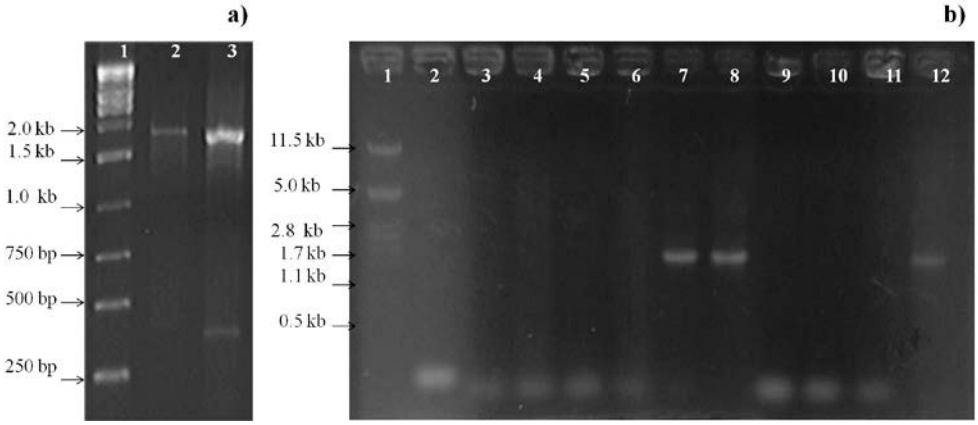

Fig. 1: a) Invertase gene fragments amplified by PCR, from the xerophilic *Aspergillus niger* GH1. Lane 1: molecular marker, lane 2 GH1strain and 1ane 3: Aa20 DNA (positive control). b) Plasmid DNA from transformed *E. coli* cells confirmed by PCR. Lane 1: molecular marker, lane 2: blue clone; 3-12: Plasmidic DNA from positive clones. Electrophoresis was done using 1 % (w/v) agarose gel, then gel was stained using ethidium bromide. Note: digestion of the plasmid DNA with *EcoRI* was realized (data no shown).

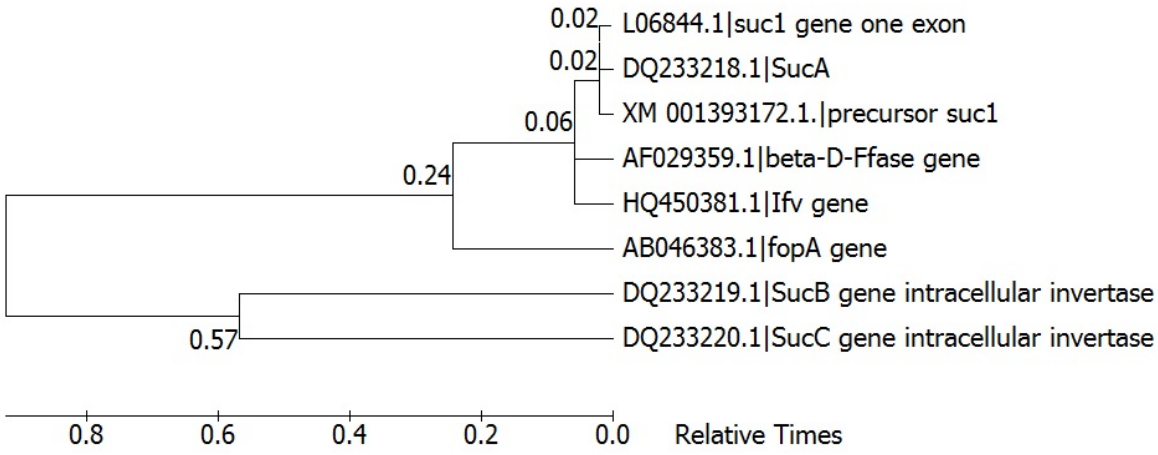

Fig. 2: Phylogenetic relationship between invertase gene sequences from *A. niger* strains reported in GenBank and invertase gene sequence from *A. niger* GH1. NJ model with 1000 bootstrap re-sampling and Tamura 3-parameter substitution model was employed previously for construction of evolutionary history, which was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 2042 is shown. The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1664 positions in the final dataset.

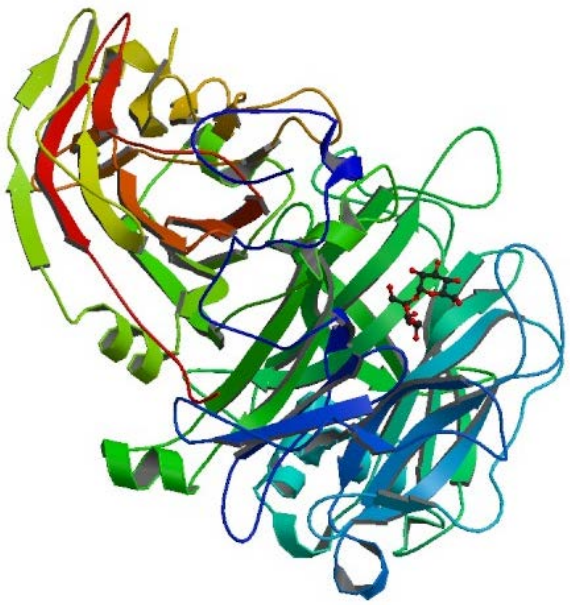

Fig. 3: Tridimensional structure of fructosyltransferase from *A. japonicus* (PDB code 3LDK) using sucrose as ligand.

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